Quantifying Western Blots Using Fiji (ImageJ)

Protocol is Based on personal experience, Luke Miller's protocol <http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/>, The University of Queensland Diamantina Institute's protocol <http://www.di.uq.edu.au/sparqimagejblots>, and ImageJ's Gels Submenu description <https://imagej.nih.gov/ij/docs/menus/analyze.html - gels>

Purpose: Use the imaging processing package Fiji to compare the relative abundance of protein bands of interest in a western blot.

Software: Fiji is a freeware used by researchers worldwide and provides an alternative to proprietary software. Fiji has been developed by collaborators around the world with strong desires to improve the tools available for life sciences to process and analyze data. Fiji is powered at its core by the Java open source imaging processing program ImageJ and builds on top of that many additional tools and capabilities. I have used Fiji in the context of working in four different labs, analyzing data ranging from western blots to single-molecule microscopy data.

Downloading the software

1. Go to https://fiji.sc/#download and download Fiji for the operating system you use. Follow the prompts for installation – you should be pretty clearly guided through the process.

Loading, orienting, and optimizing the image

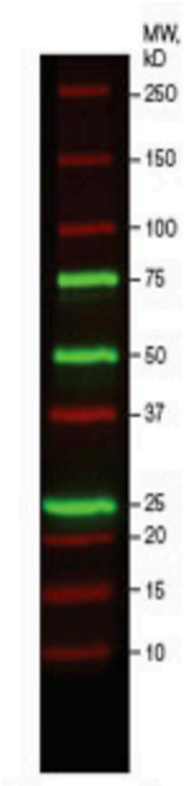
1. **Load your image** by either using file->open or simply dragging your .tif image file onto the space just under the Fiji toolbar.

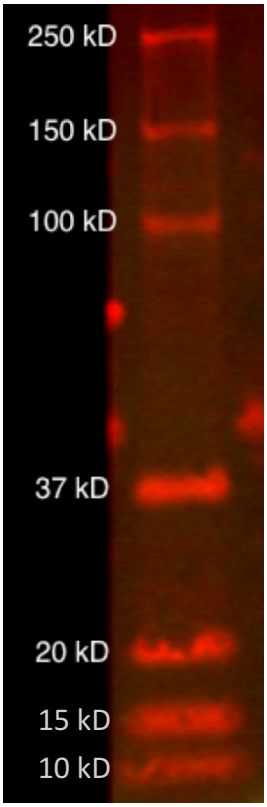
2. **Orient your image** so that the red dye front is at the bottom and the protein standard ladder is on the left (if the dye front has run off, you can identify the orientation because the protein standard bands get closer together toward the bottom… also you should already know the orientation based on notes you have taken). Changes in orientation can be made using Image->Transform->Rotate/Flip

3. **Adjust the brightness** of the image such that you can more clearly see the protein standard ladder in red, the beta-tubulin in red, and your bands of interest in green (there may be green obscuring the red beta-tubulin band - this will be taken care of in subsequent steps). Adjust the brightness by using Image->Adjust->Brightness/Contrast and moving the Maximum slider bar (the second from top) to the left until you can clearly see the ladder (don't worry about this step if your image is already bright enough).

Finding the bands corresponding to your protein of interest and to beta-tubulin

1. **Determine the molecular weight of your protein** by entering the alphanumeric code of your gene (e.g., YGR250C) into the search bar at http://www.yeastgenome.org/. The molecular weight in Daltons (Da.) will be found under the "protein" heading. Remember to convert to kilodaltons to match the units of the protein standard ladder.
2. **Add the molecular weight of your protein to the molecular weight of the triple tag**. Remember that the triple tag is made up of GFP <http://www.jbc.org/content/275/23/17556.full>,3xFLAG <http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Datasheet/7/f4799dat.pdf>, and His <https://en.wikipedia.org/wiki/Polyhistidine-tag>.The weights of these components plus your protein's molecular weight will give you your expected band size. The weights can be found in the included links, but you may find them faster with some simple Google searches.
3. **Determine the molecular weight of beta-tubulin**. This can be done by searching for Tub2 on http://www.yeastgenome.org/ and checking under the Physical Details heading.
4. **Refer to the protein standard ladder to find your bands of interest**. Note the differences between the "expected ladder" (the first image below) and the actual ladder (the second image below). Green bands are lost in our images (they may actually show up as faint red bands). Remember to refer to the WT lanes to determine which green bands are non-specific.





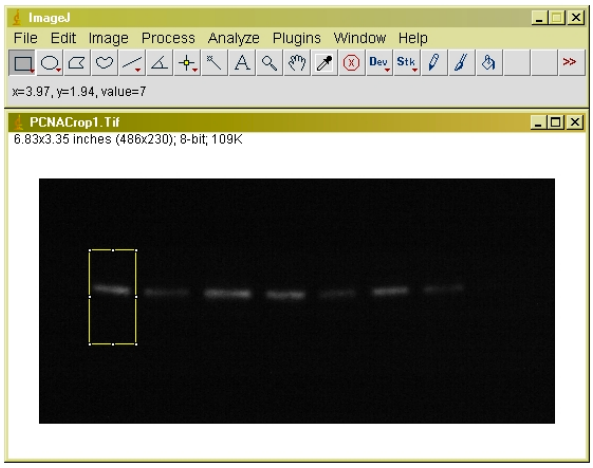
Quantifying your bands of interest

1. **Split the channels of your image** by using Image->Color->Split Channels. Your image has now been split into 3 gray-scale 8-bit images. Delete the blue channel. The red channel corresponds to the red signal from the combined image and the green channel to the green signal from the combined image. Using the method detailed above, re-adjust the brightness for each image again if need be.

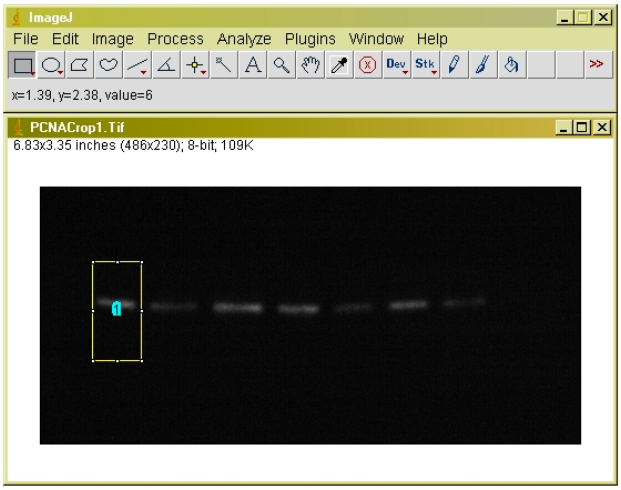
   (note, the majority of the following text and images has been copied from the University of Queensland protocol – the images will be slightly different from what you will actually see when using the software but still illustrate the concepts just fine)

   - **Do steps 2-8 first for the green channel image and the band in each lane corresponding to your gene of interest. Then repeat the process for the red channel image and the band in each lane corresponding to beta-tubulin.**

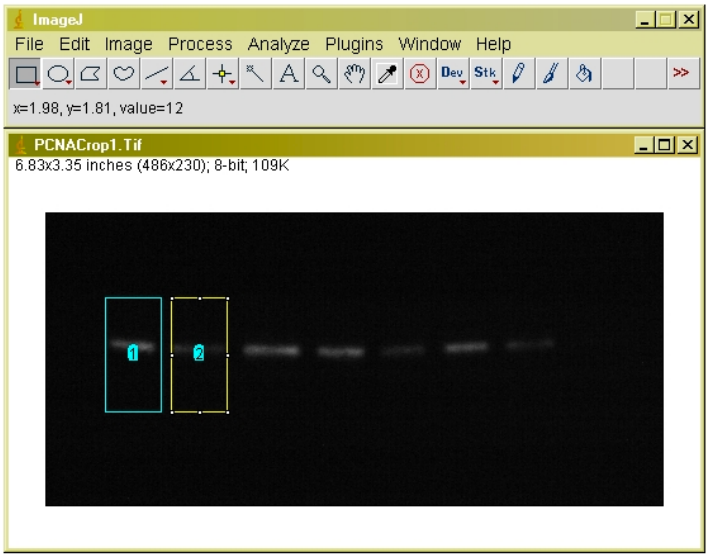
2. **Select a rectangular area around the first band** using the "Rectangular Select" tool. The area selected should be as wide as the widest band on the blot and taller than it is wide.



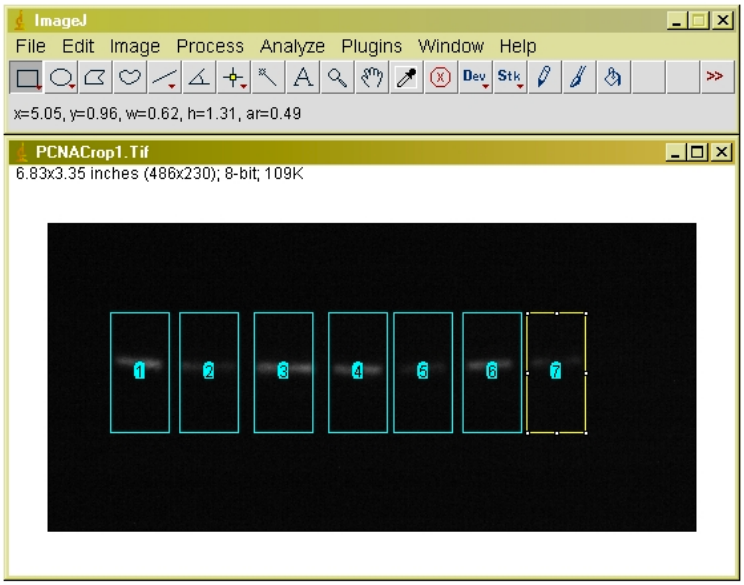
3. Press "CTRL" "1" (Command+1 for Macs) to mark this as the first band. The selection box should be yellow with a box with the number "1" inside, indicating that it is the first band selected.



4. Move the mouse cursor so that it sits over the box and changes into an arrow. Drag the box so that it sits over the next band and press “CTRL” “2” (Command+2 for Macs) to select the next band.

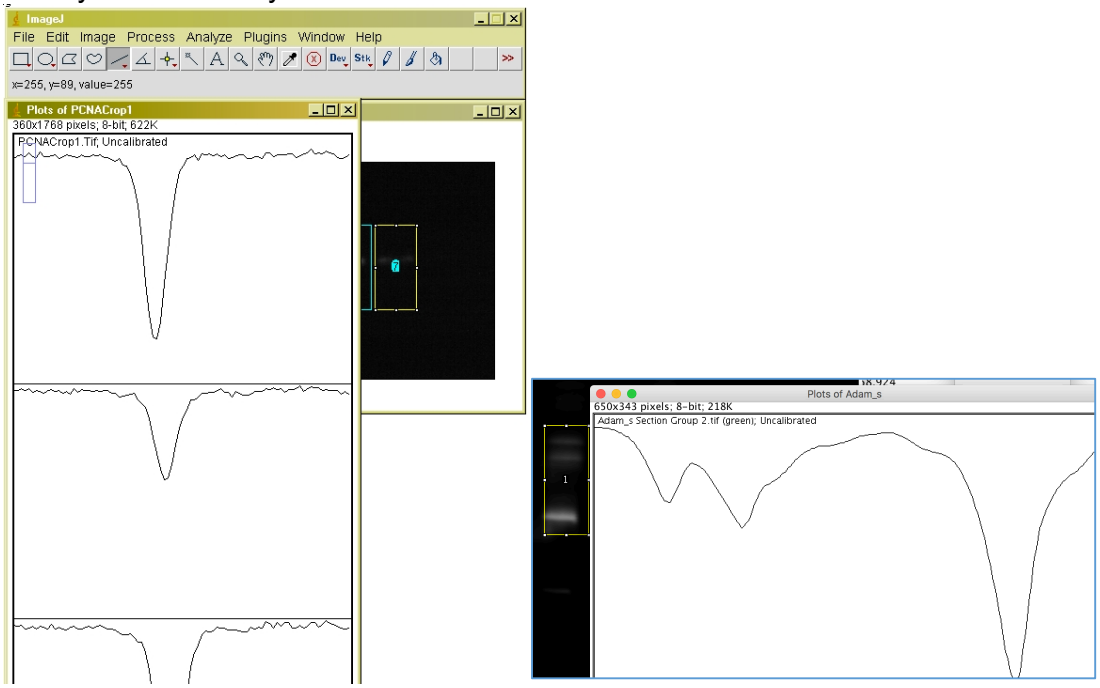


5. Repeat this step for each of the bands, pressing **“CTRL” “2” (Command+2 for Macs)** for each subsequent band (you will have to start over if you prematurely press Ctrl/Command+3). With each selection, a small box should appear in each of the selection boxes indicating the number of the band (i.e., 1-7 in our example).

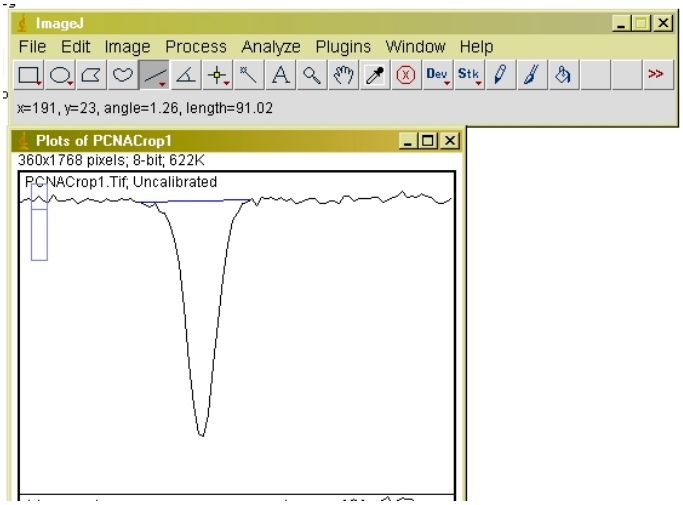


5. Once all of the bands have been selected, press “CTRL” “3” (Command+3 for Macs). This brings up another image which shows histograms indicating the intensity of each of the bands (the larger the histogram, the brighter the band). There should be a histogram for each of your bands – you may need to select the scroll button (illustrated as a hand) and drag the image down to see all of them (scrolling with a laptop touchpad works fine too). You should see something like the first image below.

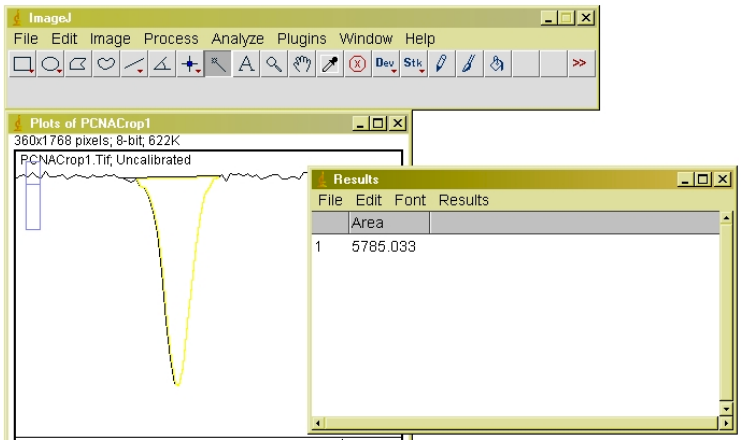
   If you have multiple bands in a selection box, they will show up as different dips in the histogram. The left-most dip corresponds to the top-most band in the selection box and the right-most dip to the bottom-most band in the selection box – you will see histograms like the one on the bottom-right below. Notice 3 distinct dips corresponding to each band. You should only perform the following steps on the dip that corresponds to the band you are actually interested in.



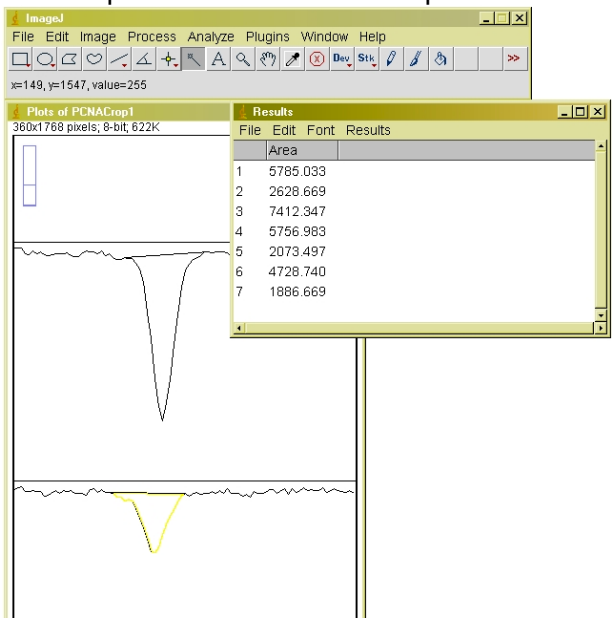
6. Starting with the histogram for the first band, select the “draw line” button and draw a line across the top of the histogram from where it first begins to drop steeply until where it levels out again.



7. Select the “magic wand” button and click anywhere inside the histogram (again – if there are multiple dips corresponding to multiple bands in the histogram, only do this for the dip that corresponds to your band of interest). The selected area should be outlined in yellow and a new window named “Results” should appear which indicates the intensity of the band as a numerical value – the brighter the band, the higher the number.



8. Repeat this process for each of the histograms. You should end up with a numerical value for each of the histograms representing the bands. Once complete, select “File” in the Results window and save the values as a spreadsheet (name them so you can identify whether they correspond to values for the protein of interest or to beta-tubulin). These values can then be copied into another excel spreadsheet for data analysis.



- **Repeat steps 2-8 for the beta-tubulin band in each lane of the red channel image – you will use these values to normalize the protein of interest values to account for differences in amount of lysate loaded into each well of the SDS-PAGE.**

Data Analysis

1. Copy the protein of interest and beta-tubulin values into two different columns of an excel spreadsheet. Divide each lane's protein of interest value by its corresponding beta-tubulin value. This is the normalized intensity of each band and will tell you their abundance, relative to one another.